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ORIGINAL RESEARCH

Emergence of *optrA*-Mediated Linezolid Resistance in *Enterococcus faecium*: A Molecular Investigation in a Tertiary Hospital of Southwest China from 2014–2018

Miao Yi[1]
Jiaqi Zou[2]
Jinxin Zhao[1]
Yu Tang[3]
Yaling Yuan[1]
Bingxue Yang[1]
Jinzhu Huang[1]
Peiwen Xia[1]
Yun Xia[1]

[1]Department of Laboratory Medicine, The First Affiliated Hospital of Chongqing Medical University, Chongqing, People's Republic of China; [2]Department of Clinical Laboratory, Chengdu Women's and Children's Central Hospital, School of Medicine, University of Electronic Science and Technology of China, Chengdu, People's Republic of China; [3]Department of Laboratory Medicine, Chongqing University Three Gorges Hospital, Chongqing, People's Republic of China

Purpose: To investigate the potential mechanism and molecular characteristics of linezolid-non-sensitive *Enterococcus faecium* from a tertiary hospital in southwest China and characterize the relevant plasmids.

Patients and Methods: Linezolid-non-sensitive *Enterococcus faecium* (LNSEFM) isolates collected from January 2014 to December 2018 were screened for resistant genes *23s rRNA, rplC, rplD, rplV, optrA, cfr, poxtA*, by PCR. Molecular epidemiological analysis was performed by multilocus sequence typing (MLST). The *optrA*-and-*poxtA* co-harboring strain *EFM_7150* was subjected to the whole genome sequencing (WGS) by Illumina HiSeq and Oxford Nanopore MinION.

Results: A total of 15 LNSEFM with linezolid MICs ranging from 4 to 16 mg/L were identified. About 66.7% (10/15) of isolates were linezolid-resistant. About 46.7% (7/15) of strains were positive for *optrA*. Two types of *optrA* variants (P and EYDNDM) were identified. About 13.3% (2/15) of isolates had *poxtA*. 1 harbored a L22 protein alteration (Ser77Thr). One isolate coharbored *optrA* (EYDNDM variant) and *poxtA*. There was no mutation in the gene that encoded the ribosomal protein *L3*/*L4* or the domain V of 23S rRNA. No *cfr* gene was detected. Based on WGS data, *optrA* was associated with Tn558 inserted to *radC* gene and *poxtA* was flanked by IS1216E.

Conclusion: *OptrA* is primary mechanism in linezolid-resistant *Enterococcus faecium*. This is the first report of*optrA* variants P and EYDNDM identified in *Enterococcus faecium* and *optrA*-and-*poxtA* co-harboring *Enterococcus faecium* clinically in southwest China. Besides, Tn558 and IS1216Es may play an important role in the dissemination of *optrA* and *poxtA*, respectively. The findings revealed the potential threat to nosocomial infection by *optrA* and coexistence of *optrA* and *poxtA* in *Enterococcus faecium*. Thus, clinical surveillance of linezolid-resistant *Enterococcus* is urgently needed.

Keywords: *optrA*, *poxtA*, linezolid-non-sensitive *Enterococcus faecium*, WGS

Introduction

Enterococcus is a Gram-positive opportunistic pathogen, normally residing in the gastrointestinal tracts of humans, which is regarded as the most common cause of nosocomial infections, such as meningitis, bacteremia, pneumonia, surgical wound infection, and urinary tract infection.[1] Its strong intrinsic and acquired resistance leads to resistance to a major group of antibiotics such as vancomycin. Hence,

Correspondence: Yun Xia
Department of Laboratory Medicine, The First Affiliated Hospital of Chongqing Medical University, 1 Youyi Road, Chongqing, 400016, People's Republic of China
Tel +86-23-89012742
Fax +86-23-89012513
Email xiayun12cn@aliyun.com

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a better understanding of the mechanism of resistance and transmission could support better strategies to monitor and control drug resistance.

The oxazolidinone linezolid targeted at the large (50S) subunit of bacterial ribosomes is considered as the last resort to methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* (VRE), and other multi-drug Gram-positive bacteria.[2] However, linezolid-resistant isolates have been increasingly monitored since their clinical use in 2000.[3,4] Linezolid-resistant *Enterococcus* (LRE) represents a significant threat to clinical treatment. The most common resistance mechanism is mutations in the central loop of the domain V region of the 23S rRNA gene, especially G2576T and varying copy numbers.[5] Moreover, mutation or deletion of genes that encode the 50S ribosomal subunit proteins *L3, L4, L22* and acquisition of resistance genes such as *optrA, poxtA* also lead to increased linezolid MIC.[6–8] Oxazolidinone and phenicol transferable gene *optrA* was first detected in a clinical *Enterococcus faecalis* from China in 2015.[7] Following its first report, *optrA* has also been discovered in many countries such as Colombia, Tunisia, Poland.[9–11] It is mostly reported in *Enterococcus* but also detected in *Staphylococcus sciuri, Streptococcus suis*, and other Gram-positive or Gram-negative strains.[12] *OptrA* is often located on chromosomes or plasmids and can be transmitted by mobile genetic elements such as transposons and insertion sequences.[13–15] The most recently reported *poxtA* gene, mediating resistance to oxazolidinones, tetracyclines, and phenicols, was first described in an MRSA isolate from respiratory secretion of an Italian patient.[8] Since its first report, it has also been detected in isolates from animals and humans in many countries, including Italy, Greece, and China.[8,16,17] Otherwise, *poxtA* is found more in the environment or food-producing animals than human samples and *Enterococcus faecium* has higher prevalence than *Enterococcus faecalis*.

Our previous transcriptomics and proteomics studies showed that *optrA* played an important role in linezolid-resistance *E. faecalis* at the First Affiliated Hospital of Chongqing Medical University and that sexual pheromones could promote *optrA* transmission.[18–21] However, the mechanism of linezolid in *E. faecium* is not yet clear. Therefore, the purpose of this study is to investigate the mechanism of linezolid in *E. faecium* and reveal its transmission by whole-genome sequencing. To the best of our knowledge, we monitored the emergence of *optrA*-mediated linezolid resistance in *E. faecium*. Besides, this is the first report of *optrA* variants, P and EYDNDM identified in *E. faecium*, and *optrA* and *poxtA* co-exist in the same strain in southwest China.

Materials and Methods

Bacterial Strains and Antimicrobial Susceptibility Tests

A total of 1891 *E. faecium* strains were obtained at the First Affiliated Hospital of Chongqing Medical University from January 2014 to December 2018. Excluding duplicate strains, 15 LNSEFMs were collected from six types of samples, including urine, blood, secretion, seroperitoneum, drainage, and bile. Then, they were stocked at −80°C with glycerol. Antimicrobial susceptibility tests were initially confirmed by AST-GP67 cards (BioMérieux) on the VITEK-2 Compact system (bioMérieux, Lyon, France), including linezolid (LZD), clindamycin (CLI), dalfopristin (DAF), tetracycline (TET), erythromycin (ERY), ciprofloxacin (CIP), moxifloxacin (MOX), levofloxacin (LEV), vancomycin (VAN), ampicillin (AMP), penicillin (PEN), tigecycline (TIG), streptomycin (STR), and gentamicin (GEN), and then linezolid MIC is manually reconfirmed by the broth microdilution method. All results were determined according to the CLSI guidelines,[22] and the *E. faecalis* ATCC29212 was used as a reference strain.

DNA Extraction and Molecular Detection of Mutation and Resistance Genes

Following the manufacturer's protocol, genomic DNA was extracted from bacteria cultured in the logarithmic growth phase using the HiPure Bacterial DNA Kit (Magen, Guangzhou, China). To investigate the mechanism of linezolid resistance, the mutation of *23s rRNA* ribosomal proteins *L3* (*rplC), L4* (*RplD), L22* (*rplV*) and the presence of *cfr, optrA*, and *poxtA* were identified using a previously described method.[18] Primers and reaction conditions are shown in Supplementary Table S2. All positive PCR products were sent to Sangon Biotech (Shanghai) Co., Ltd. for bidirectional sequencing and blasted against the NCBI nucleotide database. Nucleotides of 23S rRNA and amino acid sequences of *L3, L4*, and *L22* were compared with the reference *E. faecium Aus0004* (GenBank Accession No. CP003351) using DNASTAR package MegAlign (Version 7.1.0). The *optrA* sequence was compared with plasmid pE349 (GenBank Accession No. NG_048023.1).

Multi-Locus Sequence Typing (MLST)

According to the *E. faecium* MLST scheme, 7 housekeeping genes (adk, pstS, gyd, purK, gdh, ddl, atpA) were amplified using multi-locus sequence typing (MLST) for an analysis of sequences on the *Enterococcus faecium* database (https://pubmlst.org/mlst). Primers are listed in Supplementary Table S2.

Whole Genome Sequencing (WGS) and Bioinformatic Analysis

Whole-genome sequencing and bioinformatic analysis on *optrA*-and-*poxtA* co-harboring isolate *EFM-7150* were conducted by Shanghai Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). Following the manufacturer's protocol, Wizard® Genomic DNA Purification Kit (Promega) was used to extract genomic DNA. Genomic DNA was sequenced using a combination of Illumina and Nanopore sequencing platforms. For Illumina sequencing, 5 μg of genomic DNA was used for library construction. An Illumina HiSeq X Ten with 2 × 150 bp paired-end reads (Illumina) was used to sequence those libraries. For Nanopore sequencing, Covaris G-TUBE (Covaris, MA) was used for spinning 15 μg of genomic DNA to cut the genomic DNA into ~10 kb fragments, followed by magnetic bead purification and sequencing adapter connection to both ends. After removing low-quality reads, the following reads were assembled into a contig using HGAP and canu.[23] In the end, Pilon (1.23) was applied for error correction of Nanopore assembly results. Gene prediction was performed using bioinformatics software Glimmer (3.0) and GeneMarkS.[24] Each set of query nucleotide sequences was aligned with NR, Swiss-Prot and Pfam databases. Circular representation of complete plasmids sequences was visualized using the GView server.[25] And Plasmid replicons were identified using PlasmidFinder (2.1) (https://cge.cbs.dtu.dk/services/PlasmidFinder/).

Nucleotide Sequence Accession Number

Nucleotide sequences of *EFM_7150* complete chromosome and two key plasmids pEF7150-3, pEF7150-5 were collected in GenBank under accession numbers CP079927, CP079928, CP079929, respectively.

Clinical Data

Patient demographics and clinical data including patient age, sex, date, sample, wards, and antibiotic usage were collected from the hospital information system (HIS) and laboratory information system (LIS).

Ethical Approval Statements

This retrospective study was approved by the Evaluation Committee and the Biomedical Ethics Committee of Chongqing Medical University (2021–515). In light of the retrospective and anonymous nature of the study, the Ethics Committee did not require written informed consent provided by participants.

Results

Clinical Information and Antimicrobial Susceptibility Testing

A total of 15 nonduplicated LNSEFM isolates were recovered from 1891 *Enterococcus* according to VITEK-2 Compact by the BMD method (Supplementary Table S1). Table 1 shows that 15 isolates were obtained from eleven different wards, the most common is gastrointestinal surgery (n = 3) and urology surgery (n = 3), and there is only one isolate in other wards. Urine is the most common source, followed by secretion. According to clinical data, 10 patients were discharged, and 5 patients were transferred. Penicillin, aminoglycosides, carbapenems and cephalosporins were used during the treatment period.

The 15 strains of LNSEFM linezolid MIC spread from 4 to 16 mg/L, of which 5 strains were intermediary, and 10 strains were resistant to linezolid. In addition, the highest drug resistance rate is penicillin (93.3%), erythromycin (93.3%), and ampicillin (93.3%), followed by levofloxacin (86.7%), moxifloxacin (86.7%), and ciprofloxacin (86.7%). All strains are sensitive to vancomycin and tigecycline (Supplementary Table S1).

Screening of Linezolid-Resistant Mechanism and Sequence Type (STs)

The most common mechanism of linezolid resistance in *Enterococcus faecium* is *23S rRNA* gene mutation, but the presence of resistance genes was found to be the main cause in this study.[26] The molecular mechanism of linezolid resistance in 15 LNSEFM is detected in Table 1, showing that the positive rate of *optrA* gene is 46.7%, while the detection rate of *poxtA* is 13.3%. Of these, one strain carried both *poxtA* and *optrA*. The majority of LR *E. faecium* (70%, 7/10) had *optrA*. Compared with pE394, two of *optrA* proteins are wild-type. Four strains had a single point mutation T481P (P variant), and *EFM_7150* had K3E, N12Y, Y176D,

Table 1 Clinical Information and Screening of Linezolid-Resistant Mechanism

Date	Samples	Age/Sex	Wards	Outcome	Antibiotics Usage	Bacterial Type	MLST	LZD MIC (mg/L)	VA MIC (mg/L)	Mechanisms[a]		
										L22	*optrA*	*poxtA*
2014.10.03	Urine	86/male	Geriatrics	Discharge	Penicillin Aminoglycosides Carbapenems	Infection	414[b]	4	1			
2015.04.20	Blood	66/male	Urology Surgery	Discharge	Aminoglycosides Cephalosporins Carbapenems	Infection	1161	8	0.25		WT	
2015.05.22	Urine	70/male	Emergency ward	Transfer	Cephalosporins	Colonization	80[b]	4	0.25			
2015.06.15	Secretion	69/male	Vasculr Surgery	Discharge	Cephalosporins Carbapenems	Infection	8	8	1			+
2015.07.12	Urine	47/Famale	Urology Surgery	Transfer	Penicillin	Infection	117[b]	8	0.25		WT	
2015.08.08	Ascites	86/male	Gastrointestinal Surgery	Discharge	Carbapenems	Infection	1850[b]	4	0.25			
2015.08.18	Urine	74/male	Neurology	Transfer	None	Infection	78[b]	4	0.25			
2016.08.04	Drainage	78/male	ICU	Discharge	Cephalosporins	Colonization	1160	8	0.25			
2016.02.09	Urine	46/Famale	Rehabilitation	Transfer	Cephalosporins	Infection	78[b]	8	0.25			
2017.01.27	Drainage	70/male	Gastrointestinal Surgery	Discharge	Cephalosporins	Colonization	Unclassified	8	0.25		P	
2018.03.09	Urine	78/male	Urology Surgery	Discharge	Cephalosporins	Infection	Unclassified	8	0.25		P	
2018.07.20	Bile	51/Famale	Hepatobiliary Surgery	Discharge	Cephalosporins	Colonization	Unclassified	4	0.25	Ser77Thr		
2018.08.10	Secretion	61/male	Dermatology	Discharge	Penicillin	Colonization	425	8	0.5		P	
2018.10.02	Secretion	74/male	Nephrology	Transfer	None	Colonization	8	16	0.5		P	
2018.11.07	Secretion	65/male	Gastrointestinal Surgery	Discharge	Penicillin Cephalosporins Fluoroquinolones	Infection	117[b]	8	0.25		EYDNDM	+

Notes: P, T481P; EYDNDM, Lys3Glu, Asn12Tyr, Tyr176Asp, Asp247Asn, Gly393Asp, Ile622Met. [a]No mutations in genes encoding domain V of 23S rRNA or ribosomal proteins L3/L4 were found. [b]Isolates belonging to CC17 clone.
Abbreviation: ICU, intensive care unit.

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D247N, G393D, I622M (EYDNDM variant). One LR *E. faecium* had a L22 protein alteration. No isolates contained *cfr* gene and *L3*/*L4* alteration. No genetic mechanism was identified in 6 isolates (40%), among which 5 were LI *E. faecium*. As shown in Table 1, a total of 9 sequence types (ST) were identified among 15 isolates, of which 3 isolates were new ST with 1 to 2 alle mutation. Besides, 46.7% isolates belonged to CC17 clone complex.

Characteristics of Plasmids and the Genetic Environment of *optrA* and *poxtA*

The complete genome of *Enterococcus faecium_7150* was constructed using the data from the Illumina HiSeq and Oxford Nanopore MinION. The complete genome has 3,083,859 bp nucleotide and 307,877 reads with a GC content of 37.93%. The plasmids carrying *optrA* or *poxtA* are shown in Figure 1. The majority of CDSs code gene in the forward orientation are shown in Supplementary Tables S3 and S4. pEF7150-3 with the GC content of 36.91% has a size of 72,048 bp and 85 Coding sequences were identified. While pEF7150-3 is not completely identical to any other plasmid in the Gene Bank at the moment, as shown from the result of blast analysis, the region around 16,790 bp containing the *optrA* and *fexA* genes demonstrated 99.9% similarity and a query coverage of 22% to *Enterococcus avium* C674 (GenBank accession no. MH018573.1), *Staphylococcus sciuri* S49-1 *optrA* gene cluster (GenBank accession no. KX447572.1). Tn558 mediating *fexA* gene transfer was identified in pEF7150-3 and chromosome. Tn558 and *optrA* were inserted downstream of the *radC* gene (encoding a DNA repair protein). Moreover, the transcriptional regulator gene *araC* was located upstream of *optrA*.

pEF7150-5 with GC content of 36.11% is 21,754 bp in size and has 22 CDSs. It belongs to rep2 family and the incompatibility (Inc) 18 group plasmids.[27,28] pEF7150-5 shared 100% identity with a query coverage of 100% to pM16/0594 (GenBank accession no. MN831411.1), pC25-1 (GenBank accession no. MH784601.1), pC27-2 (GenBank accession no. MH784602.1), pHN11 (GenBank accession no. CP038176.1). Moreover, on plasmid pEF7150-5, the tetracycline resistance gene *tet (L) and tet (M)* were also identified. The *poxtA* gene was flanked by two IS1216E elements in the same orientation, which is responsible for horizontal gene transfer of *poxtA*, as previously described in *S. aureus* AOUC-0915, *E. faecium* Efa-955.[8,29]

Discussion

Linezolid is an effective drug for treating multidrug-resistant Gram-positive bacterial infection. However, the rate of linezolid-resistant has steadily risen in recent years, posing a threat to public health. Zyvox® Annual Appraisal of Potency and Spectrum (ZAAPS), Linezolid Experience and Accurate Determination of Resistance (LEADER), which monitor global pathogens and the changes in resistance to linezolid over time, suggest that the rates of linezolid-non-sensitive were 0.70% and 0.74%,

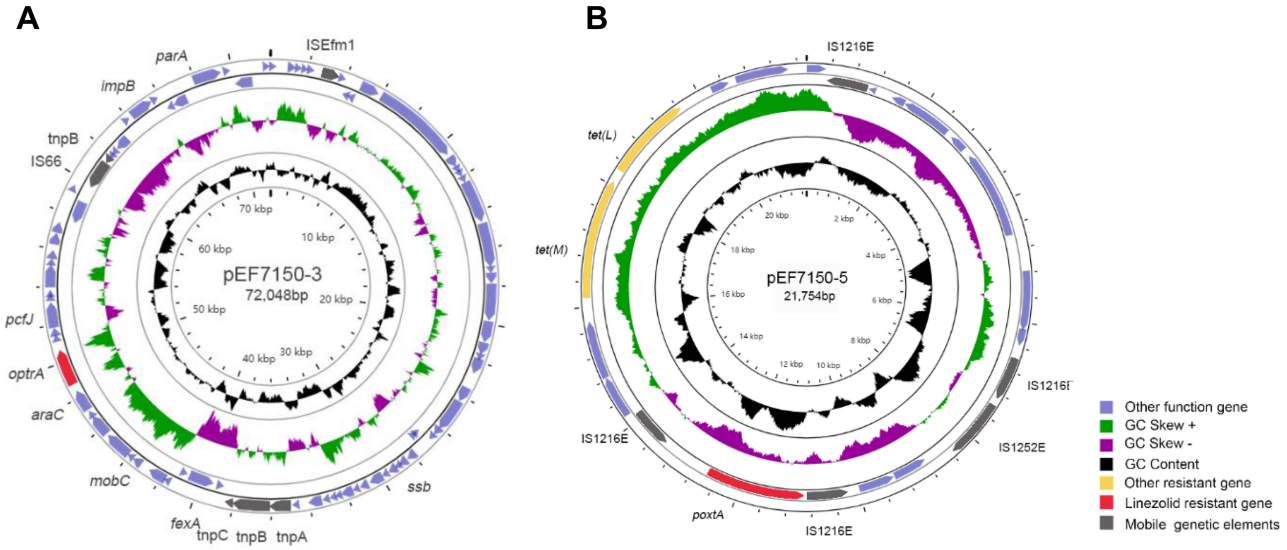

Figure 1 Structure of two resistant gene plasmids in E. faecium_7150. (**A**) Structure of the optrA-carrying plasmid pEF7150-3. (**B**) Structure of the poxtA-carrying plasmid pEF7150-5. The peripheral circle represents CDS, and arrows indicate the CDSs and their transcription directions. The second and third circle shows GC skew and GC content respectively. The purple square refers to other function gene. The yellow square refers to other resistant gene. The red square refers to linezolid resistant gene. The grey square refers to mobile genetic elements.

respectively.[30,31] In our study, the 5-year prevalence rate of linezolid-resistant *E. faecium* in our hospital was 1.9%, which is higher than that of China (1.0%).[32] Most strains (93.3%) display a multidrug-resistant phenotype (resistant to at least three antimicrobial categories), but all strains are susceptible to vancomycin. It should be noted that nearly half of the isolates belong to CC17 clone that has posed a potential threat to public health, resulting in restricted treatment options worldwide. On antibiotics usage, infected patients had no history of linezolid use during hospitalization, indicating that LNSEFM infection was not associated with linezolid use.

It is well known that mutations in the domain V of the 23S rRNA are the most common mechanism of linezolid resistance among *E. faecium*, and *optrA* appears to be almost ubiquitous among the linezolid-non-susceptible *E. faecalis*.[26] Surprisingly, we did not find 23S rRNA mutations but a high prevalence of *optrA*, which is at odds with the Sentry data.[33] This discrepancy may be explained by geographical variation. Almost all resistant isolates, except intermediates, have resistance genes. Nonetheless, we fail to find any previously described mediated genes in linezolid resistance in one isolate (MIC 8mg/L). We speculate that the observed phenotype could be related to cell wall thickness or biofilm formation.[34] Although it has been shown that *L22* protein mutation could reduce linezolid sensitivity by interfering with binding sites, this mutation is uncommon in *Enterococcus*.[6] Ser77Thr was detected in one LR *E. faecium* in our study, but the relationship between *L22* protein mutation and linezolid MIC remains unclear. Increased copy number of 23S rRNA gene resulted in increased resistance expression in the previous study,[35] but there is no evident correlation between *optrA* variants and oxazolidinone mic in *Enterococcus*.[36] Cai et al revealed that different *optrA* variants and their genetic context have the potential to regulate linezolid MIC at a variety of levels.[37] It is still important to investigate other *optrA* variants and oxazolidinone resistance level. We identified new types of *optrA* variants, P and EYDNDM in *E. faecium*. P variant was distributed in *Clostridium difficile* and *Campylobacter jejuni* and EYDNDM variants were found in *Enterococcus faecalis, Staphylococcus aureus, Staphylococcus sciuri*, Enterococcus avium.[12] Given that the relationship between *optrA* variants and linezolid MIC remains unclear, novel *optrA* variants in *E. faecium* is of concern.

To the best of our knowledge, mobile genetic elements contribute significantly to the transmission of resistant genes.[38] Tn558 carrying *fexA* gene is located on both the chromosome and the plasmids in *EFM_7150*. It integrates *optrA* through the *radC* gene in pEF7150-3, resulting in a similar genetic environment to many isolates, such as *E. avium* C674 isolated from the stool samples of healthy populations in Hangzhou and *S. sciuri* isolated from Sichuan province and Guangdong province.[37,39,40] Notably, Fan et al revealed that *optrA* can exist in methicillin-resistant coagulase-negative staphylococci including *S. sciuri* S49-1.[39] Clinicians need to be alert to the prevalence of *optrA* in other superbugs such as VRE.

The recently discovered *poxtA*, a member of the ABC-F proteins family, shares the homology of 32% with *optrA*. *PoxtA* was detected more often in the natural environment than in the clinical setting. The IS1216E-*PoxtA*-IS1216E segment in our study is similar to *S. aureus* AOUC-0915, and clinical *E. faecium* from Italy, Spain, indicating that the genetic background of *poxtA* is relatively single.[8,16,41] In addition to the conjugative plasmid pE035 detected in China, *Enterococcus* harboring both the *optrA* and *poxtA* genes were also found in the environment and human samples from Pakistan, Ireland, Spain, France[17,42–45] Unexpectedly, there does not seem to be any obvious synergistic effect when *optrA* and *poxtA* coexist. Because the MIC of *EFM_7150* is not only similar to *optrA* or *poxtA* alone isolates in our study but also to *E. faecium* C10004 isolated from Spain (8mg/L).[44] Furthermore, it is higher than strain isolated from Shanghai (0.5mg/L).[46] Despite the emergence of co-existence of *optrA* and *poxtA* worldwide, limited genetic context was identified and we hope to obtain more genomic data to explain whether there are other mechanisms or negative regulation.

Last but not the least, these results must be taken cautiously and with some limitations in mind. First, since it included just one center and the relative small number LNSEFM, the results of this study may deviate from other sets of studies. Second, the evidence that only one isolate co-harboring *optrA* and *poxtA* is not sufficient. Additional sequencing results that reveal the genetic environment will be more convincing. Nonetheless, our findings indicate the emergence of *optrA* in *Enterococcus faecium*. Furthermore, *optrA* and *poxtA* can coexist in clinical settings and may be transmitted through MGEs. This is real-world clinical experience and providing a useful information for enterococcal infection.

Conclusion

In summary, this research revealed the emergence of *optrA* in *E. faecium*, and the prevalence of *optrA* gene is higher than that of *poxtA* in *Enterococcus faecium* from 2014–2018 in our hospital. MGEs, particularly Transposons and insertion sequences may contribute significantly to the dissemination of *optrA* and *poxtA*, respectively. Although linezolid is currently effective in the treatment of enterococcal infections, advanced monitoring of changes in the resistance mechanism of linezolid is needed in the future.

Abbreviations

LNSEFM, linezolid-non-sensitive *Enterococcus faecium*; LR, linezolid resistant; LI linezolid intermediate; MGEs, mobile genetic elements.

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Disclosure

The authors report no conflicts of interest in this work.

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